Cellular water and elemental content during stages of apoptosis : investigation by a cryo-correlative nano-imaging approach (Fluorescence/STEM)

F.Nolin[1], J. Michel[1], L. Wortham[1], P. Tchelidze[2], V. Banchet[1], N. Lalun[2], C. Terryn[3], D. Ploton[2]

1Laboratoire de Recherche en Nanosciences (EA 4682), Université de Reims Champagne Ardenne, France

2CNRS UMR 7369, Université de Reims Champagne Ardenne, France

3Plate-forme PICT IBISA, Université de Reims Champagne Ardenne, France

frederique.nolin@univ-reims.fr; Téléphone : 03 26 82 35 86

1. INTRODUCTION

Cell shrinking, known as apoptotic volume decrease (AVD), is a structural hallmark of apoptosis. It is now well documented that cell death is not initiated by shrinkage but more probably by fluxes of several ions (more particularly Na^+, K^+ and Cl^-). Thus, these fluxes modify the concentrations of ions that reach specific levels required both for activation of apoptosis and for each step in its progression. Also, fluxes of ions could generate flux of water and more particularly a loss of water at the origin of cell shrinking. The most recent methods for quantification of cell volume and of water content during apoptosis are limited to the study of entire individual cell and not of their organelles. In cell physiology it is known that most of the water molecules contained within organelles hydrate all macromolecules and are essential, with ions, to their folding and activity[1]. Recently, we simultaneously quantified water and ions at nanoscale within organelles of control and stressed cancerous cells by applying our correlative light and cryo-Scanning Transmission Electron microscopy (cryo-STEM) method [2], [3], [4]. In the present work we address the variations of water and ions concentrations within the different organelles during the main steps of apoptosis by using this correlative method.

2. RESULTS

2.1 Materials and methods

a) Time-lapse experiments : To image chromatin dynamics and mitochondrial depolarization during apoptosis induced by Actinomycin-D (AMD) 500 ng/mL, HeLa cells stably expressing histone H2B tagged with GFP (H2B-GFP) were stained with rhodamine ester (TMRE). Observation was performed with a LSM 710-NLO confocal microscope equipped with a 63x Plan-apochromat 1.4 NA oil objective. Two-photon excitation at 850 nm with a CHAMELEON femtosecond Titanium-Sapphire Laser was used to generate GFP and TMRE fluorescence with limited phototoxicity and photobleaching. Differential Interference Contrast (DIC) imaging was simultaneously performed with a specific detector for transmitted light to investigate morphological changes of the cells and of their main compartments (organelles in the cytoplasm, nuclei, nucleoli). Z-stacks of 80 slices were simultaneously acquired in three channels (GFP, TMRE and DIC), each 5 minutes for 7h. Each z-stack was then processed with Amira® and Imaris® software to perform surface or volume rendering images of GFP and TMRE fluorescence at each time-point and to quantify volume of chromatin and total intensity of TMRE.

b) Immunolabeling : To correlate both chromatin and nuclear reorganization during stages of apoptosis with cytochrome-c diffusion, Caspase 3 activation and PARP cleavage, HeLa-H2B-GFP cells were treated with AMD (500 ng/mL) during 7h and fixed. Cells were labelled with anti-cytochrome, anti-cleaved PARP, anti- activated caspase 3 antibodies. Z-stacks of 80 optical sections were acquired with a LSM 710 confocal microscope by using a 63x Plan-apochromat 1.4 NA oil objective to image either sequentially or simultaneously the different labelings (chromatin, cytochrome-c, activated caspase 3 and cleaved PARP). DIC imaging was simultaneously performed with a specific detector for transmitted. Each z-stack was then processed with Amira® to obtain a surface or a volume rendering image of one, two or three fluorescent markers.

c) Targeted nano analysis of water and ions in cell compartments using cryo-correlative microscopy : We performed quantification of water and of elements concentrations by using our cryo-correlative microscopic approach coupling fluorescent imaging for identification of H2B-GFP tagged chromatin and STEM imaging to perform nano quantification of water by dark-field imaging and of elements by EDX spectrometry ([2], [3], [4]). Our detailed method is found in [4] and here it will be described briefly. Apoptosis was induced by treating HeLa-H2B-GFP cells with AMD (500 ng/mL) during 7 hours. Cells were then cryofixed by rapid plunging into liquid ethane without any cryoprotectant or chemical fixation. Vitreous sections 85 nm thick were collected on formvar-carbon coated London finder grids and directly transferred in a GATAN 626 EM cryo-holder which was inserted in a home-made cryostage for fluorescence imaging. This cryostage allowed to maintain cryosections at – 171°C and to record high-quality images by using a Zeiss Axioscope Vario A1 equipped with a 50x objective (0.55 NA; 9.1 mm working distance). Nuclei of interest were then imaged and localized relatively to letters and

numbers of pin marked on the grid. The cryo-holder then was transferred into the electron microscope JEOL 2100 FEG STEM (JEOL Company) and the cryo-sections were freeze-dried. The grid was placed in exactly the same position as in the light microscope by imaging the central delta letter of the grid and by adjusting their position by using translation and rotation functions of the STEM. After this adjustment, nuclei identified by fluorescence imaging were easily found by STEM imaging. Images were then recorded for measurement of water concentration (see [4] for details of the method) and for identification of cell compartments in which elements were quantified by EDX spectrometry. Identification of cell compartments relies on two parameters: their fluorescence status in the nucleus and their morphology in the cytoplasm. In the nucleus, condensed chromatin corresponds to highly fluorescent areas whereas surrounding nucleoplasm corresponds to areas without fluorescence. In the cytoplasm, morphology of mitochondria was easily identified due to their well-preserved cristae whereas areas outside mitochondria and containing no identifiable structures were identified as cytosol. The clear identification of these four compartments facilitated the drawing of Regions Of Interest (ROIs). Thus, elements such as nitrogen (N), oxygen (O), sodium (Na), magnesium (Mg), phosphorus (P), sulphur (S), chloride (Cl), potassium (K), were quantified and their concentration were calculated in mmol/L due to the knowledge of water concentration in the ROI investigated.

2.2 Improving the sequence of both mitochondria and nuclei modifications during stages of apoptosis. Time-lapse microscopy of TMRE labelled H2B-GFP HeLa cell lines allowed the identification of 5 stages during apoptosis. Cytochrome-c diffusion arose during stage 1. Mitochondrial depolarization arose suddenly, was complete in 10 to 15 minutes and was caspase 3 independent. During that stage, the swollen nucleus contained a large quantity of cytochrome-c but no marginated condensed chromatin. Cell shrinking began suddenly when mitochondrial depolarization was almost complete after stage 1. Then, the characteristic reorganization of chromatin allowed the definition of stages 2 to 5 and was concomitant to a regular decrease of the volume of the nucleus that reached 40 % of its initial volume at stage 5.

2.3 Water quantification. We found that apoptotic cells from stage 1 to 5 contained 10 to 15% more water in all compartments. However, cells not engaged in apoptosis (stage 0) contained around 5 to 10 % more water than apoptotic cells.

2.3 Elemental content. During stage 1, mitochondria and nuclei were characterized by strong increase in $[Na^+]$ and $[Cl^-]$ and decrease in $[K^+]$. During stage 2, mitochondrial $[Na^+]$ and $[Cl^-]$ decreased to the same value as in cytosol. During stage 3 to 5, $[K^+]$ regularly decreased in all compartments whereas $[Na^+]$ and $[Cl^-]$ regularly increased. Finally drug tolerant cells (stage 0), that never entered in apoptosis, contain higher water content, higher $[K^+]$ and lower $[Na^+]$ and $[Cl^-]$ than apoptotic cells.

3. CONCLUSION

As the aim of our study was to quantify the contents of water and of elements during each step of apoptosis by ultrastructural cryo- analysis, it was essential to identify these steps precisely in order to avoid the complexity of data interpretation due to cell population heterogeneity and asynchronous onset of apoptosis. To reach that goal, we first described the sequence of nuclear events relatively to the sequence of functional events arising at the onset of apoptosis: cytochrome-c diffusion, loss of mitochondrial potential and Casp-3 activation/ PARP cleavage. Then, by applying correlative cryo-imaging, we used fluorescence of chromatin on ultrathin cryo-sections to identify the stages of apoptosis and we performed a targeted ultrastructural cryo-analysis of water and elements contents within the main cell compartments: cytosol, mitochondria, condensed chromatin and nucleoplasm. This allowed us to evidence the strong variations in ions content from one stage to another one. More particularly, we evidenced strong increase of mitochondrial and nuclear [Na+] and $[Cl^-]$ at the onset of apoptosis.

REFERENCES

[1] Ball P. *Chem Rev*, **108**, 74–108, (2008)
[2] Nolin F et al. *J Struct Biol* , **180**, 352–361, (2012)
[3] Nolin F et al. *CMLS*, **70**, 2383-2394, (2013)
[4] Nolin F et al. *Meth. Mol. Biol.*, **1228,** 145-158, (2015)

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